THE MECHANISM OF METAL-ION ACTIVATION OF ENZYMES

STUDIES ON ENOLASE

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BO G. MALMSTRÖM

ALMQVIST & WIKSELLS BOKTRYCKERI AB

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INAUGURAL DISSERTATION

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By due permission of the Philosophical Faculty of the University of Uppsala, to be publicly discussed in the Lecture Hall of the Institute of Chemistry, May 26, 1956, at 10 a.m., for the degree of Doctor of Philosophy. In addition to the present survey, the communications numbered 24, 29, 33, 39, 42, 43, and 60 in the list of references will be publicly discussed.

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Introduction

Trace Substance–Enzyme Hypothesis

It has been known for almost one hundred years that all biological forms require small amounts of certain metals for normal growth and function (1). However, it is not until recently that an understanding of the biochemical basis of this need has begun to emerge. From the work of H. von Euler and Warburg on the chemical nature of cozymase (2), it became clear that vitamins may function by being part of coenzymes, and it was then natural to assume that the other important class of biological trace substances, the indispensable trace metals, also partake in the operation of enzymic systems. This concept was greatly strengthened by the early identification of Fe as a constituent of certain respiratory enzymes (3) and of Zn in carbonic anhydrase (4). Consequently, in 1941 Green (5) formulated the trace substance–enzyme hypothesis: "Any substance which occurs in traces in the cell and which is necessary in traces in the diet or medium must either be an essential part of some enzyme or the enzyme itself." This hypothesis has now received such ample confirmation that it may be regarded in the nature of a theory.

The identification of a large number of metalloenzymes has stimulated research on their mechanism of action, and progress in this area of investigation has been summarized in a number of recent reviews (6–12). However, in most cases the emphasis has been on enzymes containing firmly bound metals, while information on the mechanism of action of the so-called metal ion-activated enzymes has been more scanty. Since the present survey will deal only with the latter group, it is necessary first to discuss the operational basis for distinguishing between different types of metalloenzymes.

Nomenclature and Classification of Metalloenzymes

There exists a great deal of confusion concerning the nomenclature of metalloenzymes as evidenced by the review articles already quoted (6–12). Here, the term *metalloenzyme* is used for any enzyme which *requires* the presence of a metal ion for activity, i.e., whose activity is zero in the absence of the metal, regardless of the strength of interaction with the enzyme. Vallee (12) reserves this name for enzymes containing strongly bound metals and introduces the term metal-enzyme complexes for enzymes with dissociable metal ions. Since the difference is only one of stability constants and not necessarily of mechanism of action, it appears preferable to the present author that the term "metalloenzyme" includes both categories. However, it is still convenient to make a distinction between the two groups, if for no other reason because their study requires quite different experimental approaches:

I. *Enzymes with strongly bound metals* can be isolated without loss of the metal, which is present in a stoichiometric ratio, i.e., an integral number of metal ions per mole of protein. The addition of metal ions is not necessary for obtaining maximum activity but this may instead lead to inhibition. The specific activity, referred to mole of enzyme protein, is constant. These enzymes may be regarded as conjugated proteins in which the metal ion is the prosthetic group or part of the prosthetic group. Examples of this type of metalloenzymes are the cytochromes and carbonic anhydrase. Fe, Mo, and Zn are the most common metals in this group of enzymes.

II. *Enzymes with dissociable metal ions* lose activity during purification but this can be restored by addition of the proper cations. The specific activity per mole of enzyme protein is not constant but a function of the concentration of metal ion. In this case the metal ion may be regarded as the coenzyme. This terminology agrees with the origina definition of coenzyme given by Duclaux in 1897 as "a dialyzable substance necessary for enzyme activity" (13). However, some biochemists reserve this term for organic co-factors, such as diphosphopyridine nucleotide (DPN), while metal ions are called activators (14). To the present author, it would appear better to use the word "activator" for substances that increase the activity of an enzyme without being necessary for its action; but due to the prevalence of this usage, enzymes with metal-ion coenzymes will be said to be metal-ion activated also in the present survey. In the following, the discussion will be limited to this group, which includes such enzymes as enolase, some peptidases, and many phosphokinases. Mg^{++} and Mn^{++} are the most common metal-ion coenzymes.

Since the difference between the two groups of metalloenzymes suggested here is one of degree and not of kind, the border is obviously not a rigid one. Particularly Cu enzymes, e.g., ascorbic acid oxidase (15), are of an intermediate character.

Some authors, e.g. Williams (8), claim that the metal ion-activated enzymes are only "somewhat inactivated" when the metal ions are removed from the protein; in other words, the metal ions are supposed to be true activators rather than coen-

zymes. However, there are no critical experiments supporting this statement while the indispensability of the metal ion is well documented in many cases, as will be shown herein.

Historical Aspects of Metal-Ion Activation of Enzymes

To the best of the author's knowledge, the first claim of metal-ion activation of an enzyme (phosphatase) was made by Erdtmann (16) in 1928. However, his experiments were of necessity rather crude and did not throw any light on the mechanism of activation. More decisive information was obtained in the 1930's from work on proteolytic enzymes and on arginase. The first report of metal-ion activation of a proteolytic enzyme was published by Johnson *et al.* in 1936 (17). Similar observations on arginase had been described by Hellerman and Perkins in 1935 (18).

Hellerman (19) was the first one to formulate a mechanism of activation by suggesting the formation of a metal ion–enzyme complex which binds the substrate by coordinate linkages with the metal ion. However, the experimental data presented by Hellerman (pH-activity curves) give only slight support to his hypothesis which must be regarded as just a reasonable guess. More definite evidence in favor of one aspect of the mechanism was obtained in 1942 from kinetic studies of Warburg and Christian (20), who showed that the activation of enolase by Mg^{++} can be interpreted in terms of the mass-action law by assuming the active enzyme to be a dissociable complex between protein and metal ion. The applicability of this type of kinetic treatment also to a great many other enzymes was demonstrated by Emil Smith and his associates in a large series of investigations on the metal-ion activation of several peptidases (21). Smith also extended Hellerman's ideas by suggesting that the substrate is bound to the enzyme at one point by chelate formation.

Criticisms against Smith's studies have been raised [see, for example, Ref. (6)] on the ground that the enzymes were used only in partially purified or crude form. However, recently two of the peptidases have been obtained as homogeneous preparations (22, 23) and the major properties concerned with metal-ion activation previously studied on cruder products have been fully confirmed. In the opinion of the present author, a more serious criticism is the fact that the conclusions drawn about the mechanism of activation are based solely on activity measurements while no direct study of the complexes involved has been made. Smith has suggested that the fact that the activation is a slow process indicates the formation of a complex with the enzyme, but the present author has pointed out that the slow process actually cannot be the rate of combination of the metal ion with the protein (24). The formation of metal ion–substrate complexes, Smith (21) concluded from spectrophotometric measurements. However, Gilbert *et al.* (25) showed that the spectral changes recorded by Smith are due to the combination of the metal ion with O_2 rather than to complexing with the peptide. Furthermore, spectral changes alone can only give a qualita-

tive demonstration of the presence of a chelate, but they do not necessarily give an indication of the strength of interaction; in fact, with some chelating compounds the most weakly bound ion induces the largest spectral change, as shown by Schwarzenbach (26). Consequently, there is no valid experimental demonstration of the complexes postulated in Smith's mechanism. However, a study of the interaction of the activating ions with the different components (enzyme, substrate, buffer) of the reaction system appears to be a very important complement to kinetics in testing the applicability of this hypothesis, since there are alternative mechanisms that could account for the activation kinetics. One such alternative was suggested by Najjar (27) who postulated the primary formation of a metallo-substrate, and Robbins *et al.* (28) later demonstrated that this type of mechanism can account for the characteristics of the Mg^{++} activation of pyrophosphatase. Undoubtedly, more esoteric mechanisms consistent with the kinetic data could also be formulated.

In view of the above considerations it seemed desirable to study the activation of a typical metal ion-activated enzyme, available as a well-defined protein, by both kinetic and thermodynamic measurements. Comparisons between the two sets of data could then show which complexes are of importance in the reaction mechanism. Such an approach has been used by the present author in an investigation of the metal-ion activation of enolase, and in the following the main results of this study will be summarized.

The Metal-Ion Activation of Enolase

Materials and Methods

Enzyme.—The enzyme was purified and crystallized from brewer's yeast following the method of Warburg and Christian (20). The purity of the product was checked by activity measurements, absorption spectra, electrophoresis, and ultracentrifugation (29). These data indicated that the protein obtained was identical with the enzyme of Warburg and Christian (20), the physical characteristics of which has been studied by Bücher (30).

Recently it has been found (unpublished experiments) that another electrophoretic component possessing enolase activity can be obtained from yeast extracts. However, this has not been used in any of the studies described here. It is possible that it is derived from the enolase of Warburg and Christian (20) by some unknown modification of the protein; this question is being investigated further.

Substrate.—DL-2-phosphoglyceric acid (PGA) was used as substrate in all kinetic measurements. It was synthesized by the method of Kiessling (30) and purified as described by Warburg and Christian (20).

Other reagents.—Only analytical-grade (p.a.) reagents were used. These were

purified by dithizone extraction (29, 32), except in the case of activating metal ions which were used in such small concentrations that purification was not necessary. Deionized water (29, 32) was used in making up all aqueous solutions. These were stored in polyethylene bottles.

Analytical methods.—The methods used for the determination of Mg, Mn, and Zn have been described (29, 32, 33). P was determined by the method of Allen (34) and N by Kjeldahl analyses.

Determination of the interaction of enzyme with metal ions or substrates.—Klotz (35) has recently discussed the methods available for studying the binding of small ions to proteins. For the direct measurements of the interaction between enolase and other components of the reaction system, most of these could be excluded as either impractical or inaccurate. Thus, only three separate techniques were tested experimentally, namely electromotive force measurements with membrane electrodes (36), polarographic reduction (37), and equilibrium dialysis (38). While all three approaches proved feasible, equilibrium dialysis was found to be the method of choice.

The dialysis cells used have been described (29). Two important sources of error are involved in the dialysis technique, namely the Donnan effect and possible binding of the small ion or molecule to the dialysis membrane. In the studies with enolase, the former was eliminated by the use of a high enough ionic strength ($\mu = 0.056$ in most cases) (29) while the latter was corrected for by control dialyses without protein (29, 39).

Determination of the interaction of substrates with metal ions.—While the most accurate and reliable method for the study of metal chelates is the potentiometric measurement of pH (7), this was not suitable in the present case for several reasons: Free PGA is unstable since it is a strong enough acid (31) to cause autohydrolysis of the phosphate ester linkage. Also, to allow comparisons with the kinetic measurements it was desirable to determine the "apparent dissociation constants" (33) in the presence of buffer, which would counteract any changes in pH due to chelate formation. Instead, the ion-exchange method (7, 40) was chosen for measuring the interaction of PGA with activating metal ions.

The theoretical basis of this method has been treated by other authors (7, 40, 41) and the experimental tests of its applicability and validity in the present case have been summarized (33). The cation-exchange resin used was the Na form of Dowex 50. The adsorption isotherms for the different cations were determined, and on the basis of these, the concentrations were chosen to fall within the linear portion of the isotherms, which facilitates calculations (33). The use of low concentrations also had the advantage that the metal ions did not contribute appreciably to the overall ionic strength ($\mu = 0.056$), which could thus be kept constant.

The interaction with Mg^{++}, Mn^{++}, and Zn^{++} only was measured by the ion-exchange method. However, in studying the activation specificity it is of interest also to study the complexes with non-activating ions. For this purpose, the change

in ultraviolet absorption of phospho(enol)pyruvic acid (PPA) on addition of metal ions (20) was utilized (42). With this method, larger concentrations of metal ions were necessary as compared to the ion-exchange method. Thus, the ionic strength varied during the titration, especially with the weakly bound Mg^{++}, and less good constants were obtained (42).

Measurement of enolase activity.—The enzyme activity was measured by the optical method of Warburg and Christian (20) in all kinetic experiments. In the studies of activation and inhibition kinetics at constant temperature, pH, and solvent composition (29, 33, 39), a Hilger "Uvispek" spectrophotometer without thermostating arrangement was used, and the activities (a) were calculated from the spectrophotometric data by the formula of Warburg and Christian (20). However, this formula was found not to be strictly valid (24), and in recording the changes in kinetics due to varying temperature and pH (24, 43), the initial velocities were measured directly (24). For this purpose, a Beckman DU spectrophotometer with thermospacers was employed. In some recent studies with varying temperature and solvent composition, not previously described, this was provided with an energy-recording adapter coupled to a potentiometric recorder with a 1-sec. response, and with a thermostatic mixing apparatus for enzyme and substrate. (Details of this experimental arrangement will be published together with Dr. E. W. Westhead, Jr.)

Kinetics of Metal-Ion Activation

Warburg and Christian (20) showed that the dependence of the activity (a) on the concentration (M) of Mg^{++} at constant enzyme and substrate concentration can be expressed by the following equation:

$$a = \frac{a_{max} M}{K + M} \tag{1}$$

where K is the hypothetical dissociation constant of the activator–enzyme complex and a_{max} the asymptotic limit of a when M approaches infinity. However, this equation was found to be inadequate in interpreting the activation kinetics with Mn^{++} and Zn^{++}, since high concentrations of these ions inhibit the enzyme (29). By assuming that the metal ions interact with two different sets of binding sites on the enolase molecule, and that combination at the second set leads to inhibition, an equation was derived (29) which accounts for the experimental data:

$$a = \frac{k K_2 M}{(K_1 + M)(K_2 + M)} \tag{2}$$

where k is a constant (representing the theoretical value of a_{max} in the absence of inhibition), and K_1 and K_2 represent the hypothetical dissociation constants for the

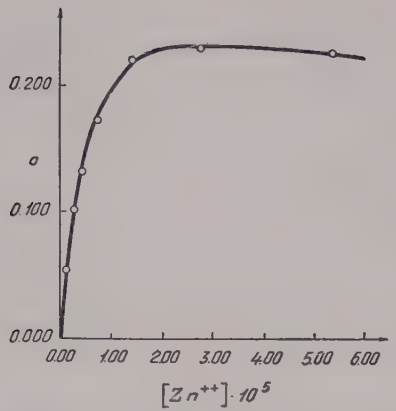

Fig. 1. Experimental points and theoretical curve, Eq. (2), for the influence of the concentration of Zn^{++} on the activity (a) of enolase in 0.025M phosphate buffer, pH 6.8, at 22°.

two types of complexes. As an example of the application of this equation, the activity data from an experiment with Zn^{++} activation, together with the theoretical curve, are given in Fig. 1. The estimated values of K_1 and K_2 for the different activating ions are summarized in Table I.

TABLE I

Kinetic Constants, Eq. (2), for Metal-Ion Activation of Enolase in 0.025 M Phosphate Buffer at pH 6.8 and 22°.

Metal ion	K_1 moles/l	K_2 moles/l
Mg^{++}	1.8×10^{-3}	—
Mn^{++}	3.8×10^{-5}	1.2×10^{-3}
Zn^{++}	5.0×10^{-6}	2.0×10^{-4}

When these studies were initiated, it was assumed that the Mn^{++} and Zn^{++} activation could be described in terms of Eq. (1). In testing the applicability of this equation, it is conveniently written in the form suggested by Lineweaver and Burk (44):

$$\frac{1}{a} = \frac{K}{a_{max}} \times \frac{1}{M} + \frac{1}{a_{max}} \tag{3}$$

Eq. (3) shows that if a^{-1} is plotted against M^{-1}, a straight line should be obtained. A Lineweaver-Burk plot with some early data (45) on Mn^{++} activation is given in Fig. 2, which seems to justify the use of Eq. (1). However, when the values of K and a_{max} are calculated from the slope and intercept in Fig. 2, the experimental data can be seen to deviate slightly from the theoretical activity-concentration plot (dashed curve, Fig. 3). These deviations may well be judged to be within the experimental error, but this is apparently not the case, since the application of Eq. (2) yields a theoretical curve (solid curve, Fig. 3) with a much better fit to the experimental points.

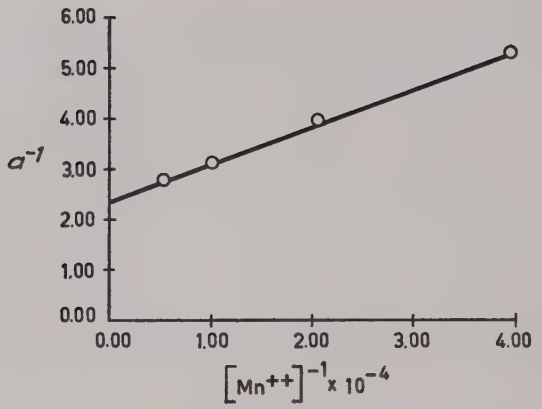

Fig. 2. Lineweaver-Burk plot, Eq. (3), for the activation of enolase by Mn^{++} in 0.04*M* phosphate buffer, pH 6.8, at 22°.

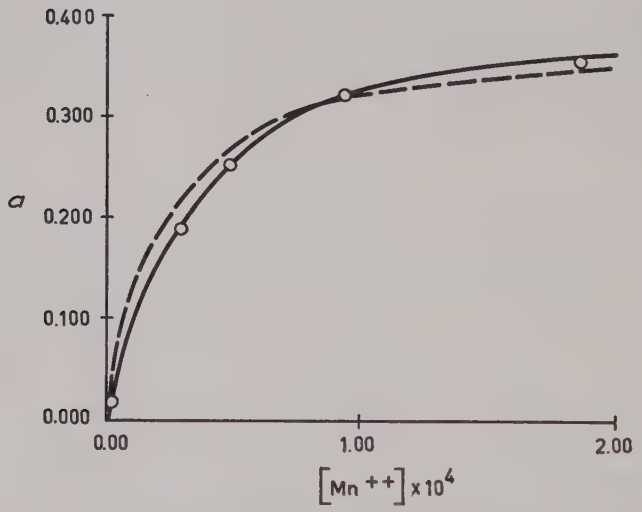

Fig. 3. The influence of the concentration of Mn^{++} on the activity (a) of enolase in 0.04*M* phosphate buffer, pH 6.8, at 22°; theoretical curves based on Eq. (1) (dashed curve) and on Eq. (2) (solid curve).

These considerations are included here because they give an indication of the accuracy of the kinetic data. They also show the danger of carrying out kinetic experiments over a limited concentration range in which case significant features of the mechanism may easily be missed. It is true that the fit of the data to Eq. (2) may be somewhat fortuitous, since the concentration range used was always relatively narrow because of the insolubility of metal phosphates. However, the main purpose here was to get a good estimate of K_1, and other reasonable mechanisms of inhibition tested [cf. Ref. (46)] lead to equations giving similar values of K_1 compared to those

obtained by Eq. (2) (unless the inhibitory reaction is assumed to be of high order with respect to the metal-ion concentration) while Eq. (1) gave considerably lower values (e.g., with the data in Figs. 2 and 3, Eq. (1) gave $K_1 = 2.5 \times 10^{-5}$ mole/l and Eq. (2), $K_1 = 4.4 \times 10^{-5}$ mole/l).

It has been suggested (29) that the inhibition at high activator concentrations is due to the electrostatic effects of activating ions bound to nonspecific sites. Another reasonable interpretation of the author's data, taking into account the hydrolysis of the activating ions, has been put forth by Hagen and Jackson (47).

In the theoretical treatments of the present author, such phenomena as cation hydrolysis and activity coefficients less than unity have been ignored since they are difficult to make corrections for in the complex systems studied. Consequently, true thermodynamic constants are not obtained, but this does not exclude the comparison of constants determined by independent methods, such as activation kinetics and equilibrium dialysis, as long as the experiments are carried out under identical conditions of medium composition (pH, ionic strength, nature and concentration of buffer, etc.).

Two medium effects can, however, be corrected for, at least approximately, namely those of phosphate buffers and substrate (24). These components increase K_1, probably by removal of the activating ion due to complex formation. In the case of the substrate, this can also in part account for the inhibition at high substrate concentrations (24, 29). The constants in Table I were determined at a PGA concentration of $2.4 \times 10^{-3}M$, where k, Eq. (2), is relatively constant (29).

An implicit assumption in the derivation of Eq. (2) is that the activity of the enzyme is zero in the absence of activating ions. However, when the dialyzed enzyme is mixed with substrate without any added activating ion, a slight activity is obtained (29, 42), which is also true of most other metal ion-activated enzymes studied (8). As has already been pointed out, this fact led Williams (8) to claim that the metal ion is not necessary in the action of metal ion-activated enzymes. In the case of enolase, a more reasonable interpretation appears to be that the residual activity is due to traces of Mg^{++} still present in the reaction medium, as evidenced in several ways. The dithizone extraction used in purifying reagents does not remove Mg^{++} impurities, and the residual activities usually found (about 5 % of the maximum activity) (42) would be caused by $10^{-5}M$ Mg^{++}, which would be obtained if as little as 0.005 % Mg were present in the buffer reagents. Furthermore, increasing phosphate concentrations decrease the residual activity (42), and small concentrations of a strong chelating agent, such as ethylenediaminetetraacetate, cause it to vanish (42). Studies on the pH dependence of K_1 for Mg^{++} (24) also indicate that the residual activity is due to trace impurities, since it is found to decrease with pH while K_1 increases. This decrease in the residual activity is not caused by the decrease in a_{max}, Eq. (1), with pH, which effect can be compensated for by increasing the enzyme concentration. Thus, if the enzyme concentration is adjusted to give the same a_{max} at all pH values, the residual activity is still zero at pH 5 while it equals about 5 %

of a_{max} at pH 7, which clearly shows that the decrease in the residual activity with pH is due to its effect on K_1, not on a_{max}. These experimental findings taken together provide strong evidence for the indispensability of the metal ion in the enzymic reaction. In the case of another enzyme, pyruvic phosphopherase, an activating ion (K^+) has also been shown to be indispensable by Kachmar and Boyer (48). It may well be that there are no enzymes of the type suggested by Williams (8) since failure to show complete loss of activity is most likely due to the use of reagents and glassware which are not metal-free. In addition, many claimed activations are probably false. In some cases where impure enzyme preparations are used, a removal of inhibitor by the metal ion may be involved. In other cases, stabilization rather than activation may occur. Such false "activations" have been found with carbonic anhydrase (49), and the stabilization of enzymes by metal ions has been demonstrated in several cases (50–52).

The type of kinetic measurements reported here are the only data available for metal ion-activated enzymes other than enolase [cf. Ref. (12)]. However, the agreement between experimental points and a theoretical curve is only a necessary but not a sufficient condition for establishing the validity of a given mechanism. As has already been pointed out (see *Introduction*), there are alternative mechanisms that could account for the activation kinetics. While more decisive information may be obtained by studying the kinetics at varying substrate concentrations [cf. Refs. (24, 28)], a better approach appears to be the use of critical experiments by a method independent of reaction rate data. Thus, in the case of enolase, the measurement of the interaction of the metal ions with enzyme alone and with substrate alone could unambiguously rule out the major alternative activation mechanism, namely the formation of metallo-substrate (27), as shown in the following sections. The success of this approach suggests its use in other cases as well, since it cannot be assumed *a priori* that the mechanism of enolase activation applies to all metal ion-activated enzymes.

The Interaction of Enolase with Activating Ions

The most extensive data on the interaction between enzyme and metal ions have been obtained with Zn^{++}, and only the binding of this ion will be discussed in any detail here. The Mg^{++} binding is too weak to allow accurate measurements. While the Mn^{++} binding is considerably stronger, the analytical methods for this ion are inaccurate in the low concentration range where activation occurs (29). Furthermore, Mn^{++} appears to be bound to more sites than Zn^{++} (29, 45) which complicates an analysis of the data. While Mn^{++} is ordinarily the stable form of Mn (53), complications may still arise from oxidation, since complex formation may affect the redox potential. For these reasons, it soon appeared desirable to limit the studies to Zn^{++}, which, in addition, is the most strongly bound of the activating ions and can be determined by a very sensitive method (29, 32).

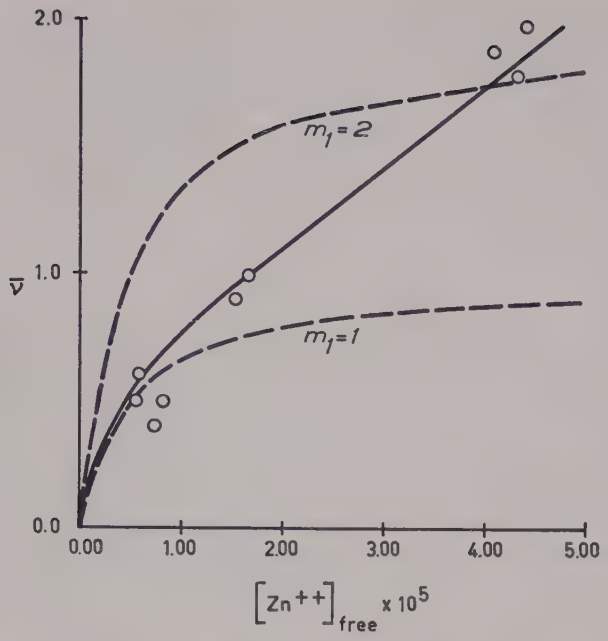

Fig. 4. The binding of Zn^{++} to enolase in 0.025M phosphate buffer, pH 6.8, at 4°. The dashed curves represent specific binding calculated from Eq. (5), assuming $K_1 = 5 \times 10^{-6}$ mole/l, and $m_1 = 1$ and 2, respectively.

Fig. 4 shows the binding of Zn^{++} to enolase in 0.025M phosphate buffer at pH 6.8 and 4°. If activation involves the binding of the ion to the enzyme, the kinetic data necessitate that several (at least two) sets of binding sites are involved. Assuming that these do not interact so that the decrease in the strength of binding of successive ions is due to statistical factors alone, it should be possible to describe the binding curve in Fig. 4 by an equation of the following form (35):

$$\bar{\nu} = \sum \frac{m_i M}{K_i + M} \tag{4}$$

where $\bar{\nu}$ is the average number of ions bound per molecule of enzyme, m_i is the number of sites in the ith set of identical sites (e.g., the number of imidazole groups), and K_i the overall dissociation constant for the interaction with these sites.

Since all proteins interact with Zn^{++} to some extent (35), the interpretation of the binding data in terms of activation is made much more difficult than in the case of enzymes with strongly bound metals, where the nonspecifically bound ions can be removed without dissociating the active complex. The only possible approach appears to be a direct comparison between the data in Figs. 1 and 4. It can be seen that the binding is appreciable in the low concentration range where activation occurs,

showing that complex formation with the enzyme protein is a possible activation mechanism. Furthermore, the kinetically determined dissociation constants (Table I) indicate that the binding of Zn^{++} to enolase is stronger than the nonspecific interaction with other proteins, which occurs primarily through imidazole groups (54). It may, therefore, be possible to separate specific and nonspecific binding; for this purpose, Eq. (4) is conveniently written in the following form:

$$\bar{\nu} = \frac{m_1 M}{K_1 + M} + \sum \frac{m_j M}{K_j + M} \tag{5}$$

where the first term represents specific binding. Using the value of K_1 determined from activation kinetics (5×10^{-6} mole/l, Table I), curves for the specific binding when m_1 equals 1 and 2, respectively, have been drawn in Fig. 4 (dashed curves). The specific binding must be less than the total binding; thus, the only possible value of m_1 is 1, since the experimental uncertainty in $\bar{\nu}$ is no larger than ± 0.1 (24). Consequently, the kinetic results can be fully accounted for by assuming that activation involves the binding of one metal ion per molecule of enzyme. As the other most reasonable interpretation, the formation of metallo-substrate, can be ruled out (see next section), this mechanism is the only one that is consistent with all the experimental data so far available.

Vallee (12) states that, in what he terms "metalloenzymes" (see *Introduction*), the metal ion is present in a stoichiometric ratio while this may not be the case in the metal ion-activated enzymes. However, the present data suggest that also in the latter group, a stoichiometric amount of metal is bound to the protein, the main difference between the two categories being the relative stability constants of the complexes.

The value of m_1 indicates that there is only one active site on the enolase molecule. No definite information on the groups involved in binding the activating ion is available, and this is one of the main problems remaining to be elucidated. The pH dependence of activation and binding implicates the involvement of an imidazole group (24). The stability of the complex indicates chelate formation, and the other site involved may be a carboxyl group (42). However, definite information can only be obtained by chemical techniques. It may be possible to break down the relatively stable Hg^{++} enolase without dissociating the metal, and studies of this nature are being planned.

The Interaction of PGA with Activating Ions

The experiments on the interaction of PGA with Mg^{++}, Mn^{++}, and Zn^{++} showed that 1:1 complexes are formed in the concentration ranges studied (33). The dissociation constants, in the presence (K_d') and absence of phosphate (K_d), at pH 6.8, $\mu = 0.056$, and a temperature of 23°, are given in Table II.

TABLE II

Dissociation Constants of Metal-Ion Complexes of PGA, in the Presence (K_d') and Absence (K_d) of Phosphate Buffer, at pH 6.8, $\mu = 0.056$, and 23°.

Metal ion	K_d' moles/l	K_d moles/l
Mg^{++}	2.9×10^{-3}	1.3×10^{-3}
Mn^{++}	1.5×10^{-3}	4.4×10^{-4}
Zn^{++}	1.0×10^{-3}	3.8×10^{-5}

Obviously, K_d' is in no case identical with the hypothetical dissociation constant determined from the kinetic measurements, which would seem to exclude the primary formation of metallo- substrate as the mechanism of activation. However, in the case of Mn^{++} and Zn^{++}, where the concentration of PGA is large compared to that of the metal ion, it may be argued that a concentration of metallo-substrate sufficient to saturate the enzyme may be reached at an activator concentration where the major portion of PGA is still free. If this were the case, however, the metal ion concentration necessary to reach saturation would decrease with increasing PGA concentrations while the opposite is found to be true (24). Furthermore, if enough metal ion has been added to reach a limiting activity at a low PGA concentration, the addition of more substrate should not increase the activity further, but such an increase is actually found (24, unpublished experiments). It is thus clear that the activation process involves primary complex formation with the enzyme, not with the substrate. The substrate does, however, have a low but definite affinity for the metal ions. In view of this, the hypothesis of Smith (21), according to which the metal ion partakes in the formation of the enzyme–substrate complex, appears feasible, and its possible applicability to the enolase reaction will be considered in the following section.

The Mediating Action of the Activating Ions in the Formation of the Enzyme-Substrate Complex

If the metal ion furnishes a point of interaction between enzyme and substrate, the affinities of the separate metallo-enolases for PGA would be expected to differ since the K_d values for the three ions are different. In the cases where the equilibrium treatment (55) is applicable, the Michaelis constant is a measure of substrate affinity. In the enolase reaction, this approximation appears valid within the errors of the measurements (33, 39); therefore, the substrate kinetics in the presence of different activating ions has been studied.

Substrate kinetics.—The kinetic analysis given by Warburg and Christian (20) assumes that the activity reaches a limiting value at high substrate concentrations, as predicted by the Michaelis-Menten equation, and that the reaction then follows

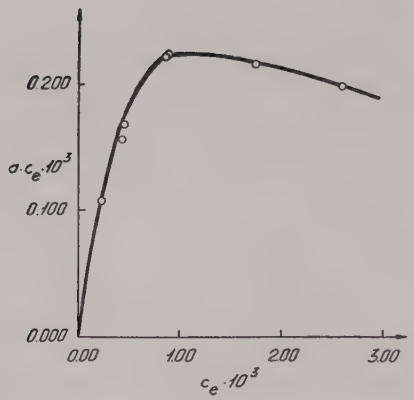

Fig. 5. The influence of substrate concentration on enolase activity in 0.025M phosphate buffer, pH 6.8, at 22° with Mg^{++} as activating ion ($[Mg^{++}] = 8 \times 10^{-3}$).

the kinetics of a reversible reaction which is first order in both directions. The data of the present author (29, 42) show that the last assumption is approximately valid, but a true saturation with substrate is never reached. Instead, high substrate concentrations were found to inhibit the enzyme, as shown in Fig. 5 which illustrates the substrate kinetics with Mg^{++} as activator (29). It was found possible to account for the variation in the initial velocity, $(dc/dt)_0$, with substrate concentration (S) at high activator concentration by the following equation [Eq. (12), Ref. (29)]:

$$\left(\frac{dc}{dt}\right)_0 = \frac{k_1 K_i E S}{(K_m + S)(K_i + S)} \qquad (5)$$

where K_m is the Michaelis constant, K_i the inhibition constant, and E the concentration of active enzyme. The data are neither extensive nor accurate enough to allow a rigorous test of the inhibition mechanism. Some possibilities have been discussed (24, 29); undoubtedly, removal of activator is one factor (24), but the mechanism is most likely complex. However, the main purpose here is to estimate K_m and, as in the case of activation kinetics, any mechanism taking the inhibition into account gives a better value than one neglecting it (except a mechanism involving a high order of S). Eq. (5) was found to be the simplest formula describing the experimental data.

The data in Fig. 5 yield a value of K_m of 1.1×10^{-3} mole/l which is very similar to K_d' for Mg^{++} (Table II) and, therefore, consistent with Smith's hypothesis. However, with Mn^{++} and Zn^{++}, approximately the same K_m values were obtained as with Mg^{++} (39). Thus, these data give no support to the bridging concept, but actually they by no means exclude it since there is no correlation between the chelating ability of a metal ion and its efficiency as a bridge in the binding of small molecules to proteins (39, 56).

Dialysis experiments.—Because of the relatively high values of K_m, accurate measurements of the binding of substrates could not be obtained by the dialysis technique (39). However, PGA was found to be bound also in the absence of metal ions, which shows that, if the metal ion partakes in the binding of substrate, it cannot be the sole point of attachment. The feasibility of the bridging hypothesis was demonstrated by model experiments in which it was shown that Zn^{++} albumin binds PGA much more strongly than free albumin (39). However, subtler functions of the metal ion in its complex with the protein may be visualized; for example, the ion may be necessary to induce a conformation change making an otherwise unreactive group, such as sulfhydryl, available. Klotz *et al.* (57) have shown that metal ions can induce slow intramolecular changes in proteins, and the data of Smith (23) show that such reactions may be of importance in the activation process [cf. Ref. (24)]. Even if the bridging concept may be applicable in the case of enolase, steric factors are also of importance in the mechanism of activation, as will be shown.

Activation Specificity and the Role of the Activating Ion in Catalysis

The specificity problem in the cation activation of enolase has been discussed rather extensively (42). It was shown that there is no correlation between the strength of binding of an ion to enolase and its ability to activate the enzyme. It is of particular interest that Mg^{++} and Ca^{++} are bound with approximately the same strengths (42), since these two ions are antagonistic in their effects on many physiological processes, e.g. nervous activity (58). Mg^{++} and Ca^{++} show about the same degree of binding also to serum albumin (36, 59). It thus appears that ion antagonism cannot be explained in terms of protein–ion affinity, but very likely opposed effects on enzyme reactions are involved [cf. Ref. (58)]. In view of the latter hypothesis, it is noteworthy that Ca^{++} inhibits Mg^{++} enolase by competing with Mg^{++} for the active site (29, 42).

The binding strength of an activating ion determines the concentration range in which activation occurs. However, the relative activity with a given activating ion is determined by some property other than complex stability (42). If the bridging hypothesis is valid, this property could be the ability to form complexes with the substrates, but this is apparently not the case since all inactive divalent cations studied interact with PGA and PPA (42). It seems more likely that the configuration of the metal ion- substrate complex is involved because of the fact that the metal ion is not the sole point of attachment to the enzyme (39). Thus, the activating ions can all form tetrahedral structures while, for example, chelates of the inactive Ni^{++} often have a square configuration (7). Differences in the properties of the substrate complexes of some activating and inhibiting ions have also been demonstrated by ultraviolet (42) and infrared (60) absorption measurements.

While various characteristics of the substrate chelates may be important in determining the activation specificity, the properties of the different metal ion–enzyme

complexes are undoubtedly also significant. It has been suggested (29, 42) that steric factors are mainly involved in determining the specificity. One basis of this idea is the fact that, for many enzymes, the activating ions all have very similar ionic radii (42). The importance of steric effects is also evidenced by measurements of the temperature dependence of the enolase reaction with different activating ions (43). More detailed kinetic studies at different temperatures, particularly low ones (0° to −30°), have been initiated in collaboration with Dr. E. W. Westhead, Jr. On the whole, this investigation is not concerned with the activation mechanism and, consequently, falls outside the scope of the present survey. However, some of the results appear related to the steric effects of the activating ion and will, therefore, be briefly discussed.

As has been stressed by Hinshelwood (61), valuable information on the relative importance of energy and entropy (steric) factors in a reaction is obtained by introducing substituents into the reactants, or by altering the solvent in which the reaction occurs. In enzymic reactions, the lability of proteins makes the first approach difficult in most cases, but in metal ion-activated enzymes not possessing absolute specificity, the substitution of different activating ions can easily be made. With enolase, replacing Mg^{++} by Mn^{++} or Zn^{++} increases the activation energy for the breakdown of the enzyme–substrate complex (43). However, the drop in the reaction rate is not as large as would be predicted from the higher energy requirement, since this is partly compensated by a more favorable steric factor. This shows that the bond strengths as well as the geometry of the transition state is influenced by the nature of the activating ion [cf. Ref. (61)]. In addition, the Mg^{++} enzyme is "inhibited" at around 0°, while the Zn^{++} enzyme offers no evidence of inhibition in the same temperature range. The data reported earlier [Fig. 1, Ref. (43)] show a large spread at the temperatures where inhibition occurs, but with the new technique of adding the enzyme (see *Methods*) reproducible results were obtained. To explain the differences in energies of activation, steric factors, and low-temperature inhibition, it has been suggested that the active site can have several distinct configurations, these being influenced by the temperature and the nature of the activating metal ion (43).

With the Mg^{++} enzyme, it has been found that effects similar to those obtained by substitution of the activating ion may result from changes in solvent composition. Thus, increasing concentrations of glycerol give rise to elevated activation energies, partially compensated by more favorable entropy factors. At the same time, increasingly lower temperatures are necessary to cause inhibition. As an example of the experimental results, the temperature dependence of the enolase reaction in 27.4 % glycerol with Mg^{++} as an activator is illustrated in Fig. 6; for comparison, the curve obtained earlier (43) in the absence of glycerol is also given. The kinetic parameters (43) at 22° have been calculated: $k = 38$ sec.$^{-1}$, $\Delta F^{\neq} = 15{,}200$ cal. × mole^{-1}, $\Delta H^{\neq} = 17{,}600$ cal. × mole^{-1}, and $\Delta S^{\neq} = +8.2$ cal. × mole^{-1} × degree^{-1}. These data show that the forces and steric conditions in the neighbourhood of the

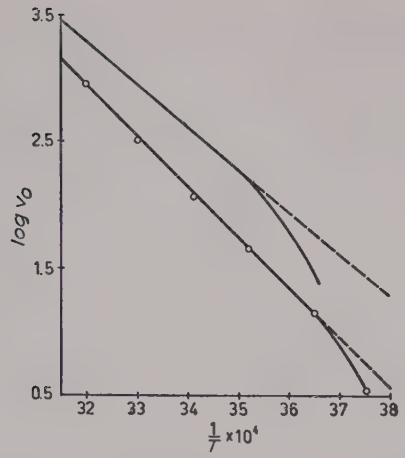

Fig. 6. The temperature dependence of the enolase reaction in the presence of glycerol. Composition of substrate mixture: $2.4 \times 10^{-3} M$ PGA, $0.025 M$ phosphate buffer, pH 6.8, 27.4 % (w./w.) glycerol, and $1 \times 10^{-2} M$ Mg^{++}. The curve without experimental points was recorded in the absence of glycerol (43).

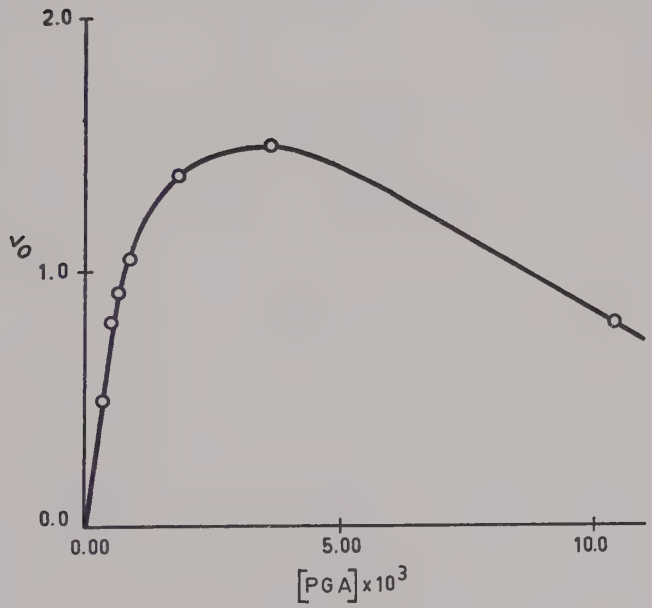

Fig. 7. The influence of substrate concentration on enolase activity in $0.025 M$ phosphate buffer, pH 6.8, at 0° with Mg^{++} as activating ion ($[Mg^{++}] = 1 \times 10^{-2}$) in the presence of 27.4 % (w./w.) glycerol.

reaction center approximate those of the Zn^{++} enzyme in the absence of glycerol. The mechanism of the glycerol effect is not established, but it appears probable that factors such as changes in solvation and lowering of the dielectric constant are involved. Since in any chemical reaction, energy and entropy factors are always in opposition, a compromise between them governing the reaction rate, it is obvious from the data given here that the steric effects of the metal ions may to a large extent determine the activation specificity.

An interesting finding is the fact that, while the rate of the enolase reaction can be varied by several orders of magnitude by changes in the temperature and solvent, the Michaelis constant is practically constant. As an example, the substrate kinetics in 27.4 % glycerol at 0° is illustrated in Fig. 7 (cf. Fig. 5). Under these conditions, the rate is about 100 times lower than at 22° in the absence of glycerol but K_m is only decreased by about 40 %. This offers additional circumstantial evidence to the conclusion that K_m approximates the dissociation constant of the enzyme–substrate complex rather than the ratio of the rate constants for two consecutive reactions, as found with some enzymes (62). In terms of transition state theory (63), the effect of the solvent on a reaction rate can be predicted from the changes in the thermodynamic activities of the reactants and the activated complex. Thus, it is more likely that the solvent affects the formation and dissociation of the enzyme–substrate complex in a parallel manner, rather than its formation and breakdown, since the first two reactions involve the same transition state.

It is a well-known fact that metal ions can have catalytic effects also in non-enzymic reactions (7). This has led to speculations [see Ref. (6–12, 42)] regarding the relative contribution of the metal ion and the protein in the catalytic process. However, in the opinion of the present author, these discussions are rather meaningless, since in most cases *both* the activating ion and enzyme protein are essential components of the catalyst. In the case of enolase, and many other enzymes (6–12), no catalytic activity is inherent in the metal ion alone. Thus, if the metal ion induces the configurational and electronic changes in the substrate necessary for the reaction to take place, its properties must have been greatly modified by the protein. In this regard, it is interesting that complex formation with some amino acids and peptides may greatly affect the metal ion bound (25, 64). However, even in reactions where a metal ion alone has a definite catalytic activity, e.g., the decarboxylation of oxaloacetate, the mechanism in the presence of enzyme is different from the model reaction, since the best metal-ion coenzyme has no activity alone (8). The recent suggestions of Williams (65) cannot account for the experimental findings in most cases. In fact, it does not seem that his model reactions bear any direct relation to the enzymic catalysis involving metal- ion coenzymes. The data for enolase indicate that the lowering of the activation energy involves the same factors as discussed by Eyring *et al.* (66) for enzymes in general, the function of the metal ion being to modify the protein rather than the substrate [cf. Ref. (42)].

Summary and Conclusions

1. The presence of certain metal ions has been shown to be indispensable for the catalytic action of enolase.

2. The kinetics of the metal-ion activation of the enzyme has been studied. The metal-ion coenzymes have been found to function as activators at low concentrations and as inhibitors at high concentrations. A suggested mechanism, involving binding of the metal ion to the protein, has been shown to account for the rate data. Alternative reaction schemes, as well as experimental methods for distinguishing between them, have been discussed.

3. The interaction of activating ions with enolase has been studied by equilibrium dialysis. With Zn^{++}, it was shown that a good correlation with the kinetic data was obtained by assuming that activation involves the binding of one metal ion per molecule of enzyme protein. In addition, nonspecific binding takes place; this may be related to the inhibition a high activator concentrations.

4. The pH dependence of activation kinetics and Zn^{++} binding indicates that an imidazole group of histidine furnishes one of the coordination points in the binding of the activating ion to the protein.

5. The dissociation constants of the Mg^{++}, Mn^{++}, and Zn^{++} complexes of DL-2-phosphoglyceric acid have been determined by an ion-exchange method. The complexes formed are of the 1 : 1 type. The data exclude the primary formation of metallo-substrate as the mechanism of activation.

6. The substrate kinetics of the enolase reaction has been studied. High substrate concentrations have been shown to inhibit the enzyme. This is in part due to the substrate competing with the protein for the activating ion. Equilibrium kinetics appears applicable so that the Michaelis constant is a measure of the affinity between enzyme and substrate.

7. The applicability of the bridging hypothesis of Smith to the enolase reaction has been tested experimentally in several ways. The Michaelis constant was found to be practically independent of the nature of the activating ion. Dialysis experiments showed that the metal ion cannot be the sole point of attachment between enzyme and substrate. In model experiments with serum albumin, it was shown that proteins can bind DL-2-phosphoglyceric acid by the mediation of Zn^{++}.

8. The metal-ion specificity in the enolase reaction has been studied. Mg^{++}, Mn^{++}, Fe^{++}, and Zn^{++} have been found to activate the enzyme. The maximum activities obtainable with these four ions are not the same. All other metal ions tested are inhibitory. With several ions, the inhibition has been shown to be competitive with respect to Mg^{++}.

9. Attempts have been made to find correlations between the known properties of the metal ions and their effects on the enolase reaction. All activating ions can

form tetrahedral complexes and have similar ionic radii. There is no relation between activity and the stability of the metal-ion complexes with enzyme or substrate.

10. Ultraviolet and infrared absorption measurements have demonstrated differences between the substrate complexes of some activating and inhibiting ions.

11. The temperature dependence of the enolase reaction with different activating metal ions has been investigated, and the results have been interpreted in terms of transition state theory. It is shown that the metal ion has a pronounced effect on the forces and steric conditions at the active site of the enzyme.

12. On the basis of the relatively extensive experimental material, it is suggested that the function of the metal ion in the enzymic catalysis is mainly twofold: (a) to furnish one of at least two points of interaction between enzyme and substrate; and (b) to modify the structure of the active site on the enzyme surface.

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